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REPUBLIC OF SOUTH AFRICA

EPARTEMENT VAN HANDEL
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Certificate

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- 1) South African Provisional Patent Application No. 2002/5742 accompanied by a Provisional specification was filed at the South Africa Patent Office on 18 July 2002, in the name of **Agricultural Research Council and Kynoch Feeds (Pty) Limited** in respect of an invention entitled: "Microorganism and its uses".
- 2) On 3 July 2003, an assignment of South African Patent Application No. 2002/5742 from **Agricultural Research Council and Kynoch Feeds (Pty) Limited** to **Agricultural Research Council and Kemira Phosphates (Pty) Limited** was recorded at the South African Patent Office.
- 3) The photocopy attached hereto is true copy of the provisional specification and drawings filed with South African Patent Application No. 2002/5742.

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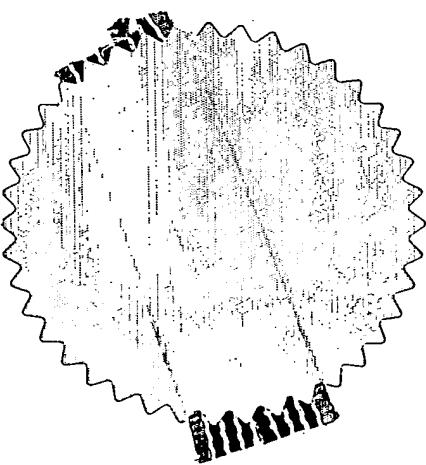
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Signed at

PRETORIA

in die Republiek van Suid-Afrika, hierdie
in the Republic of South Africa, this

29th

dag van
day of July 2003



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Registrateur van Patente

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PATENTS ACT, 1978

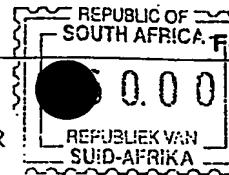
REGISTER OF PATENTS

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ASSIGNEE(S)					
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FULL NAME(S) OF INVENTOR(S)					
72	HORN, Charles Henry KISTNER, Albrecht GREYLING, Barend Jacobus SMITH, Alexandra Helena				
PRIORITY CLAIMED		COUNTRY		NUMBER	
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TITLE OF INVENTION					
54	MICROORGANISM AND ITS USES				
ADDRESS OF APPLICANT(S) / PATENTEE(S)					
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ADDRESS FOR SERVICE					REF
74	D M Kisch Inc, 66 Wierda Road East, Wierda Valley, SANDTON			P24533ZA00	
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FRESH APPLICATION BASED ON		DATE OF ANY CHANGE			

REPUBLIC OF SOUTH AFRICA
PATENTS ACT, 1978

APPLICATION FOR A PATENT AND ACKNOWLEDGEMENT OF RECEIPT
(Section 30 (1) - Regulation 22)

The grant of a patent is hereby requested by the undermentioned applicant
on the basis of the present application filed in duplicate.



Form P.1

OFFICIAL APPLICATION NO	
21	01
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FULL NAME(S) OF APPLICANT(S)	
71	AGRICULTURAL RESEARCH COUNCIL KYNOCHE FEEDS (PTY) LTD Share: KEMIRA PHOSPHATES (PTY) LTD

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03.07.2003

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TITLE OF INVENTION	
54	MICROORGANISM AND ITS USES
	THE APPLICANT CLAIMS PRIORITY AS SET OUT ON THE ACCOMPANYING FORM P2 The earliest priority claimed is
	THIS APPLICATION IS FOR A PATENT OF ADDITION TO PATENT APPLICATION NO. 21 01
	THIS APPLICATION IS FRESH APPLICATION IN TERMS OF SECTION 37 AND BASED ON APPLICATION NO. 21 01

THIS APPLICATION IS ACCCOMPANIED BY :	
<input checked="" type="checkbox"/>	1a A single copy of a provisional specification of 62 pages.
<input type="checkbox"/>	1b Two copies of a complete specification of pages.
<input type="checkbox"/>	2a Informal drawings of sheets.
<input checked="" type="checkbox"/>	2b Formal drawings of 3 sheets.
<input type="checkbox"/>	3 Publication particulars and abstract (form P8 in duplicate).
<input type="checkbox"/>	4 A copy of figure of the drawings for the abstract.
<input type="checkbox"/>	5 Assignment of invention (from the inventors) or other evidence of title.
<input type="checkbox"/>	6 Certified priority document(s).
<input type="checkbox"/>	7 Translation of priority document(s).
<input type="checkbox"/>	8 Assignment of priority rights.
<input type="checkbox"/>	9 A copy of form P2 and a specification of S.A. Patent Application. 21 01
<input checked="" type="checkbox"/>	10 A declaration and power of attorney on form P3.
<input type="checkbox"/>	11 Request for ante-dating on form P4.
<input type="checkbox"/>	12 Request for classification on form P9.
<input type="checkbox"/>	13a Request for delay of acceptance on form P4.
<input type="checkbox"/>	13b

DATED 18 July 2002

ADDRESS FOR SERVICE	
74	D M Kisch Inc 66 Wierda Road East Wierda Valley SANDTON

PATENTATION BY POST APPLICANT(S), TRADE MARKS AND COPYRIGHT	
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The duplicate will be returned to the applicant's address for service as proof of lodging but is not valid unless endorsed with official stamp.

REPUBLIC OF SOUTH AFRICA
PATENTS ACT, 1978

DECLARATION AND POWER OF ATTORNEY
(Section 30 - Regulation 8, 22(i)(c) and 33)

PATENT APPLICATION NO.	LODGING DATE	D M KISCH'S REFERENCE
21 03 2002 / 5742	22 18-07-2002	P24533ZA00

FULL NAME(S) OF APPLICANT(S)

- 71 1. AGRICULTURAL RESEARCH COUNCIL
 2. KYNOCHE FEEDS (PTY) LTD

FULL NAME(S) OF INVENTOR(S)

- 72 1. HORN, Charles Henry
 2. KISTNER, Albrecht
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 4. SMITH, Alexandra Helena

EARLIEST PRIORITY CLAIMED

COUNTRY

NUMBER

DATE

NOTE: The country must be indicated by its International Abbreviation - see Schedule 4 of the Regulations

33 -

31 -

32 -

TITLE OF INVENTION

- 54 MICROORGANISM AND ITS USES

We, 1.) Dr. H. H. Meissner; and 2.) M.C. de Villiers

hereby declare that --

We have been authorised by the applicants to make this declaration and have knowledge of the facts herein stated in the capacity of 1.) Director of Animal Nutrition and Animal Products Institute and 2.) Managing Director of Kynoch Feeds Pty Ltd. respectively of the applicants;

the inventor(s) of the abovementioned invention is/are the person(s) named above and the applicants has/have acquired the right to apply by virtue of an assignment from the inventor(s);

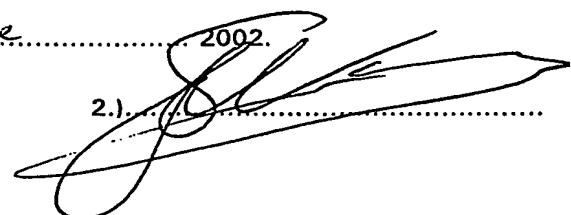
to the best of our knowledge and belief, if a patent is granted on the application, there will be no lawful ground for the revocation of the patent;

the directors and qualified staff of the firm of D M KISCH INC, patent attorneys, are authorised, jointly and severally, with powers of substitution and revocation, to represent the applicants in this application and to be the address for service of the applicants while the application is pending and after a patent has been granted on the application.

SIGNED AT Irene THIS 24th DAY OF June 2002.

1.) Dr. H. H. Meissner

2.) M.C. de Villiers



REPUBLIC OF SOUTH AFRICA

PATENTS ACT, 1978

PROVISIONAL SPECIFICATION
(Section 30 (1) - Regulation 27)

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FULL NAME(S) OF APPLICANT(S)			
71	AGRICULTURAL RESEARCH COUNCIL KNOCH FEEDS (PTY) LTD Share! KERLA PHOSPHATES (PTY) LTD <i>AANGEKERS VERVANG APPLICANTS SUBSTITUTED 03.07.2003</i>		
FULL NAME(S) OF INVENTOR(S)			
72	HORN, Charles Henry KISTNER, Albert GREYLING, Barend Jacobus SMITH, Alexandra Helena		
TITLE OF INVENTION			
54	MICROORGANISM AND ITS USES		

MICROORGANISM AND ITS USES

INTRODUCTION

5 This invention relates to a novel strain of *Megasphaera elsdenii* and its uses. This invention further relates to preparations and methods incorporating such strain. This invention also relates to feedstuffs for ruminants and a preparation and method for the prevention and treatment of lactic acidosis in ruminants.

10 BACKGROUND

Lactic acidosis

Lactic acidosis is a digestive disorder in ruminants that may occur when there
15 is a sudden excess intake of readily fermentable carbohydrates, particularly when ruminants are switched from a diet of roughage to a high-energy or energy rich concentrate diet containing a high level of starch. The disorder is characterised by an accumulation of organic acids, especially lactic acid, in the rumen (Dawson & Allison, 1988). Studies have indicated that a gross
20 imbalance between the numbers of lactic acid-producing bacteria and lactic acid-utilising bacteria, brought on by a sudden increase in the proportion of readily fermentable carbohydrates in the diet is the main cause of the onset of lactic acidosis (Slyter, 1976).

Manipulating the rumen microbial population to prevent lactic acidosis by administering material containing high numbers of lactate-utilising bacteria has been advocated for decades, but never practiced on a large scale.

- 5 Manipulations to enhance lactate utilisation within the rumen has been achieved by administering rumen fluid from an already adapted animal (Allison *et al.*, 1964; Braun *et al.*, 1992) and by administering pure or mixed bacterial cultures of lactate-utilisers (1,251,483 Wilker *et al.*, 1971; 3,857,971 Abdo & Cahilly, 1974; 4,138,498 Das, 1979; 5,380,525 Leedle *et al.*, 1991; Hession &
- 10 Kung, 1992; Robinson *et al.*, 1992; Wiryawan & Brooker, 1995).

Some of these feed additives containing live bacterial cultures have been patented (1,251,483 Wilker *et al.*, 1971; 3,857,971 Abdo & Cahilly, 1974; 4,138,498 Das, 1979; 5,380,525 Leedle *et al.*, 1991), but not commercialised
15 extensively or at all. In three of the patents (1,251,483 Wilker *et al.*, 1971; 3,857,971 Abdo & Cahilly, 1974; 4,138,498 Das, 1979) the cultures were obtained from continuous culture fermenters with an initial inoculum of rumen fluid. However, the donor animals were not necessarily adapted to a high-concentrate diet. There is also no mention of pH tolerance for any of these
20 cultures. In the other patent (5,380,525 Leedle *et al.*, 1991) the cultures were isolated at pH 5.3 either directly or indirectly after enrichment from ruminants adapted to high-concentrate diets.

The incidence of sub-acute and acute acidosis in dairy cattle

Sub-acute rumen acidosis is a common and serious health and production
5 problem in the dairy industry because dairy cows are usually fed diets containing high levels of grains. Sub-acute and acute rumen acidosis are simply different degrees of the same problem. Acute rumen acidosis is more severe and physiological functions may be significantly impaired. The affected animal is depressed and usually ataxic, off-feed, with dilated pupils and an
10 elevated heart rate. Diarrhoea will be obvious and the animal may become recumbent and die within 2 to 5 days after the insult (Nordlund, 1995). Acute acidosis is characterised by a dramatic reduction in ruminal pH (≤ 5.0), a large increase in lactic acid concentration and a large decrease in protozoa (Nocek, 1997).

15

Signs of sub-acute rumen acidosis are very different from that of acute acidosis. Modern dairy management systems of group housing or group feeding make it difficult to recognise these symptoms because individual cows with these problems will usually not be noticed within a group. Herds with sub-
20 acute rumen acidosis will present some or all of the following signs: laminitis, intermittent diarrhoea, poor appetite or cyclical feed intake, high herd cull rates for poorly defined health problems, poor body condition in spite of adequate energy intake, abscesses without obvious causes and hemoptysis (coughing of

blood) or epistaxis (bleeding from the nose). Most of these signs are secondary to acidosis and most of them do not appear until weeks or months after the initial acidosis events. Contrary to feedlot cattle, dairy cows are kept for years and the management of acidosis is therefore of importance in

5. increasing profits.

Chronic laminitis is perhaps the most consistent clinical sign of a herd with sub-acute rumen acidosis. Although the relationship between acidosis and laminitis is not completely understood, the association is widely recognised clinically and

10. demonstrated in research trials (Kelly & Leaver, 1990; Manson & Leaver, 1988; Nocek, 1997). Furthermore, most dairy managers, veterinarians and nutritionists tend to underestimate or perhaps tolerate an abnormal incidence of laminitis and lameness in dairy herds. A survey in Minnesota demonstrated a mean incidence in lameness of 15% with a range of 0 - 33% (Nordlund, Garret

15. & Oetzel, 1995). Studies in Europe have identified lameness as the third most costly health problem in dairy cows after mastitis and reproduction (McDaniel & Wilk, 1989). The management of acidosis is thus clearly of utmost importance.

A major symptom of sub-acute acidosis is decreased feed intake and

20. decreased efficiency of milk production. Sub-acute acidosis, because of difficulties in diagnosing the problem, tends to be dismissed as other problems, such as poor management, poor forage quality etc. However, it is probably the

biggest economic sink to many dairy farmers because it is omni-present, particularly in high producing dairy herds.

Because of the high incidence of nutritional and metabolic disturbance amongst
5 high producing dairy cows, nutritional strategies for improving performance with cereal based diets focus on the prevention of ruminal dysfunction by controlling acid production or by stimulating more efficient microbial growth. At present, feed additives play an important role in this regard (Hutjens, 1999). The use of yeast culture strains that specifically stimulate the growth of lactic acid utilising
10 bacteria generates much interest and a recent survey indicated that yeast cultures are being used in 33% of high producing Wisconsin herds. Results from various studies suggest that the Yea Sacc strain 84170 appears to be particularly well suited for altering ruminal fermentation and animal production when used in high lactate silages and feeds high in concentrates (Dawson,
15 1995). Production results, however, are very inconsistent. In the USA the cost for yeast culture supplementation is 4 – 6 cents per cow per day (Hutjens, 1999). Ionophores, because of their ability to prevent the growth of important lactic acid producers, can also play a role in managing sub-acute acidosis. Although the cost is relatively low (1 – 2 US cents/cow/day, Hutjens, 1999)
20 there seems to be some resistance against the use of ionophores because of a few recent cases of ionophore toxicity. Furthermore, ionophores have not been registered in the USA for use in dairy cattle diets.

Experimentally, there have been several bacteria that have potential as direct fed microbials (DFM) for ruminants, but have not been commercialised for a number of reasons. For example, *Megasphaera elsdenii* (ME) is the major lactate-utilising organisms in the rumen of adapted cattle fed high grain diets.

- 5 When cattle are shifted from high forage to high concentrate diet, the numbers of ME are often insufficient to prevent lactic acidosis. Kung and Hessian (1995) have shown that the addition of ME B 159 prevented accumulation of lactic acid during a challenge with highly fermentable carbohydrates. Robinson *et al* (1992) demonstrated that addition of a different strain of ME (407A) prevented
10 lactic acidosis in steers.

Although the costs associated with subclinical ruminal acidosis are difficult to pinpoint, the potential costs to the dairy industry are huge (Hall, 1999). Donovan (1997) conservatively estimated the cost of subclinical acidosis to
15 the US dairy industry at \$500 million to \$1 billion per year.

Elsden and Lewis (1953) first described a large, strictly anaerobic Gram-negative, fatty acid producing, non-motile coccus isolated from the rumen of sheep. However, the original isolate was lost before it had been characterised
20 phenotypically in detail. An organism resembling the original strain was isolated from the rumen contents of sheep several years later by Elsden and his colleagues (Elsden *et al.*, 1956). The characteristics of this organism did not fit the description of any known species at the time, but in view of the small

number of isolates studied, the authors refrained from assigning the organism to a new species and genus, but referred to it as LC. Gutierrez *et al.* (1959) encountered a similar organism in the rumens of bloating cattle and concluded that they fell within the definition of the genus *Peptostreptococcus*, proposing 5 the creation of a new species *P. elsdenii*. Subsequently, Rogosa (1971) demonstrated that the LC-type isolates were Gram-negative and therefore should not be included in the genus *Peptostreptococcus*. He proposed transfer of *P. elsdenii* to a new genus *Megasphaera* and the new combination *M. elsdenii*, with the isolate LC1 of Elsden *et al.* (1956) as the type strain. *M. 10 elsdenii* is a strict anaerobe found mainly in the rumen of young animals and animals receiving high-concentrate diets in which lactate fermentation is particularly pronounced. The organism has also been isolated on occasion from the faeces of humans (Sugihara *et al.*, 1974) and it ferments lactate to mainly butyrate, propionate, isobutyrate, valerate, CO₂, H₂ and sometimes trace 15 amounts of caproate (Stewart and Bryant, 1988). Since *M. elsdenii* is not subject to catabolite repression by glucose or maltose as in *Selenomonas*, which is also a lactate utiliser occurring in the rumen, its contribution to lactate catabolism is particularly enhanced subsequent to feeding of soluble carbohydrates (Stewart and Bryant, 1988).

20

USA patent 3,956,482 (Hahn *et al* 1976) discloses a method of increasing milk production in ruminants including the steps of administering to the rumen of a lactating cow acetate producing micro-organisms consisting of a mixture of

0-4% *M. elsdenii*, 30-42% *Streptococcus bovis*, 3-10% *Lactobacillus acidophilus*, 12-20% *Bifidobacterium adolescentis*, 18-44% *Bacteroides ruminicola* and 3-12% *Butyrivibrio fibrisolvens* cultured and adapted to a nutrient medium.

5

A major disadvantage of the invention disclosed in the above patent is the relatively high percentage (between 30-42%) of *Streptococcus bovis*, which together with *Lactobacillus* is the leading cause of lactic acidosis in ruminants.

10 The mixture further contains a relatively low percentage of *M. elsdenii* (0-4%) and the administration of the mixture would probably aggravate or initiate ruminal lactic acidosis rather than preventing or treating it. The mixture is further exposed to atmosphere so that most of the *M. elsdenii* perish. A mixture of microorganisms is furthermore much more difficult to control than a pure culture.

15

USA patent 4,138,498 (Das, 1979) discloses a feed additive for administration to ruminants to prevent or minimise lactic acidosis when ruminants are switched from a diet of roughage to starch, comprising a bacterial culture of *M. elsdenii* admixed with an ingestible animal feed additive. *M. elsdenii* is strictly anaerobic and a disadvantage of the feed additive disclosed in this patent, over and above the disadvantages set out below, is that the *M. elsdenii* is exposed to atmosphere, leading to a rapid decline in the amount of viable cells available in the additive.

USA patent 5,380,525 (Leedle *et al.*, 1991) discloses a biologically pure culture of *M. elsdenii* NRRL-18624 and its use in the facilitation of the adaptation of ruminants from a roughage or normal pasture to a high-energy starch-rich diet.

5 The culture suffers from the disadvantages set out below.

USA patent 5,529,793 (Garner *et al.*, 1996) discloses a mixture of lactic acid producing bacteria and a lactate utilising bacteria such a *M. elsdenii* with a dry formulation or an animal feedlot diet for improving the utilisation of feedstuffs by 10 a ruminant. A disadvantage of this invention is that *M. elsdenii* is generally strictly anaerobic and the application thereof to dry feedstuffs would result in most of the cells dying.

The applicants have evaluated the above strains of *M. elsdenii* and have 15 deducted that they are generally not suitable for commercialisation and large scale preventative treatment of lactic acidosis in ruminants because of the following disadvantages of these strains namely they are not:

- 20
- highly active and adapted to proliferate in the rumen of animals on high-concentrate diets;
 - capable of proliferating at relatively low pH values below pH 5.0 and as low as 4.5, characterised as acute acidosis;
- [Handwritten signature]*

- resistant to ionophore antibiotics commonly added to feedlot diets; and
 - capable of preferentially using lactate as a carbon source even in the presence of soluble carbohydrates such as glucose and maltose.
- 5 Further disadvantages of these strains are that, generally, they:
- have a relatively low growth rate, i.e. less than 0.938 h^{-1} ;
 - do not have the ability to grow on reducing sugars as well as on lactate;
 - have a relatively low biomass output rate, i.e. less than $0.39 \text{ g (l.h)}^{-1}$;
 - are not ionophore resistant; and
- 10 - produce predominantly propionate and butyrate and not predominantly acetate.

OBJECT OF THE INVENTION

It is therefore an object of the present invention to provide a novel strain of *M. elsdenii* and its uses, and preparations and methods incorporating such strain with which the aforesaid disadvantages can be overcome or at least minimised.

SUMMARY OF THE INVENTION

According to a first aspect of the invention there is provided a biologically pure
20 bacterial culture of *M. elsdenii* having substantially the same 16S ribosomal RNA sequence as that of the *M. elsdenii* strain deposited at NCIMB, Aberdeen, Scotland, UK under number NCIMB 41125.

According to a second aspect of the invention there is provided a biologically pure bacterial culture of the *M. elsdenii* strain deposited at NCIMB, Aberdeen, Scotland, UK under number NCIMB 41125.

5 The *M. elsdenii* strain in accordance with the first and second aspects of the invention is further characterised by its:

- ability to utilise lactate very efficiently even in the presence of sugars;
 - resistance to ionospheres;
 - relatively high growth rate;
- 10 - capability to produce predominantly acetate; and
- capability to proliferate at relatively low pH values below 5.0 and as low as 4.5.

According to a third aspect of the invention there is provided a composition for
15 facilitating the adaptation of ruminants from a roughage-based diet to a high-energy concentrate-based diet, the composition consisting essentially of the bacterial culture of the first or second aspects of the invention.

According to a fourth aspect of the invention there is provided a method of
20 facilitating the adaptation of ruminants from a roughage-based diet to a high-energy concentrate diet including the step of administering to the rumen of said ruminants an effective amount of a bacterial culture according to the first or second aspects of the invention.

According to a fifth aspect of the invention there is provided a feedstuff for ruminants comprising a carrier and an effective amount of a bacterial culture according to the first or second aspects of the invention.

5

Preferably the culture is disposed in an anaerobic container.

According to a sixth aspect of the invention there is provided a method for the prevention and/or treatment of any one or more of the following, namely
10 ruminal lactic acidosis, rumenitis, laminitis, bloat and liver abscesses, including the step of anaerobically administering to the rumen of a ruminant an effective amount of a bacterial culture according to the first or second aspects of the invention.

15 According to a seventh aspect of the invention there is provided a veterinary agent for the prevention and/or treatment of any one or more of the following, namely ruminal lactic acidosis, rumenitis, laminitis, bloat and liver abscesses, comprising an effective amount of a bacterial culture according to the first or second aspects of the invention.

20

According to an eighth aspect of the invention there is provided a preparation for the prevention and/or treatment of any one or more of the following, namely

ruminal lactic acidosis, rumenitis, laminitis, bloat and liver abscesses in ruminants comprising:

- an inoculum of a bacterial culture according to the first or second aspects of the invention; and
- 5 - a separate anaerobic growth medium,

the components of the preparation being disposed in separate chambers of an anaerobic container which are anaerobically connectable to each other, thus to inoculate the growth medium with the culture anaerobically.

10 According to another aspect of the invention there is provided a method of achieving any one or more of the following improvements in ruminants namely:

- increased milk production;
- improved feedlot performance;
- improved growth rate;
- 15 - decrease in finishing time;
- lower digestive morbidity and mortality;
- lower incidence of lactic acidosis and related diseases;
- improved feed conversion efficiency; and
- capability to feed on relatively higher concentrate diets,

20 including the step of administering to the rumen of a ruminant an effective amount of a bacterial culture according to the first or second aspects of the invention.

Preferably the culture is administered anaerobically.

According to yet another aspect of the invention there is provided a method of isolating a biologically pure culture of a superior ruminal microorganism in a relatively shorter time period than conventional methods, the method including the steps of:

- obtaining a sample of ruminal fluids; and
- cultivating the sample on a pre-selected growth medium,

the method being characterised in that a plurality of parameters selected from the group comprising growth medium constituents, dilution rate, pH, temperature anti-microbial agents, gaseous environment, redox potential, lack of nutrients and challenging organisms, are pre-selected to favour the superior rumen microorganism to the detriment of inferior rumen microorganisms.

15 The invention will now be described in more detail below with reference to the below examples and the enclosed drawings wherein:

Figure 1 is a graph of growth rates of lactate utilisers at various pH values;

Figure 2 is a graph of the growth rates (h^{-1}) of the lactate utilising isolates, on glucose medium at various pH values; and

20 Figure 3 is the phylogenetic tree of *M. elsdenii* according to the present invention.

In accordance with the present invention, organisms capable of utilising lactic acid were isolated directly from ruminants adapted to a high-concentrate diet. The objective was to select those cultures with the best combination of characteristics for the purpose of application as mass-cultured, preserved 5 inocula for prophylactic and/or therapeutic treatment of lactic acidosis.

For the lactate-utilising bacteria to be effective they must be highly active and adapted to multiplication in the rumen of animals on high-concentrate diets. The organisms should be able to multiply at pH values below pH 5.0. The 10 selected isolates should also be resistant to ionophore antibiotics commonly added to feedlot diets. Lactate should be preferentially used as a carbon source even in the presence of soluble carbohydrates such as glucose and maltose, which would be present in high proportions in high-concentrate diets.

15 METHODS

1. Animals used during isolations

Samples of rumen contents from animals with a pre-selection for lactate utilising bacteria were chosen, namely lactating fistulated dairy cows at the 20 Dairy Cow Nutrition unit, Irene, of the Agricultural Research Council (South Africa), as well as feedlot cattle of Chalmar Beef (Pretoria, South Africa) which were slaughtered at the end of their finishing periods. All the animals

- were adapted to high-concentrate diets, which increased the numbers of naturally occurring lactate utilising bacteria.

2. Sample collection and preparation

- 5 Samples of rumen contents were collected from dairy cows at about 09h00, after the cows had been fed and milked. Samples of rumen contents from feedlot animals were obtained 15-30 minutes after the animals had been slaughtered. Plastic screw-cap sample bottles were filled to capacity with rumen fluid filtered through two layers of cheesecloth. The rumen fluid was
10 transferred directly into the fermenter.

3. pH-auxostat

- A New Brunswick Scientific Bioflo 1 continuous culture system was modified into a pH-auxostat by converting the pH-dosing pump to a medium addition
15 pump. The pH was monitored with a Schott S23158 pH-electrode connected to a Digital Data Systems 302 pH-meter and titrator. A poorly buffered medium was added whenever the pH increased over the set value until the desired value was reached. The working volume of the culture vessel was 270 ml. The maximum dilution rate obtained for a given organism during auxostat cultivation
20 is a measure of the maximum growth rate of that organism during that condition.

4. Isolation of lactate utilising rumen bacteria via the auxostat

4.1 Growth conditions and medium

Filtered rumen fluid was used to fill the fermenter (270 ml) initially and the titrator activated to add sterile medium (Medium 1) to the culture proportionally to the increase in pH of the culture. Medium 1 was a semi-defined rumen fluid free medium consisting of: Na-lactate (70%), 10 g/l; Peptone, 2 g/l; KH₂PO₄ 1 g/l; (NH₄)₂SO₄ 3 g/l; MgSO₄.7H₂O 0.2 g/l; CaCl₂.2H₂O 0.06 g/l.; Vitamins (Pyridoxolhydrochloride, 4 mg/l; Pyridoxamine, 4 mg/l; Riboflavin, 4 mg/l; Thiaminiumchloride, 4 mg/l; Nicotinamide, 4 mg/l; Ca-D-pantothenate, 4 mg/l; 4-Aminobenzoic acid, 0.2 mg/l, Biotin, 0.2 mg/l, Folic acid, 0.1 mg/l and Cyanocobalamin, 0.02 mg/l); Na₂S. 9H₂O, 0.25 g/l; Cysteine, 0.25 g/l; Antifoam, 0.07 ml/l and Monensin, 10 mg/l. The Na-lactate and mineral solution were both added to the reservoir bottle and autoclaved for 60 min. The peptone was dissolved in 300 ml d.H₂O and autoclaved separately in a 1.0 l Schott bottle with a bottom outlet fitted with Quick-fit glass connections. The vitamin solution was filter sterilised beforehand as well as the two reducing agents. Following autoclaving, the reservoir bottle was gassed with anaerobic gas overnight and the other constituents added separately after cooling. The pH was adjusted to the desired value with 5N HCl.

Continuous culturing followed until a pure culture was observed microscopically. A sample was taken from the fermenter with a sterile syringe,

which was sealed and transferred into the anaerobic cabinet (Forma Scientific model 1024). One droplet of the culture was streaked out in a Petri dish containing Medium 1 solidified with 2% agar. Incubation followed at 39°C overnight and a single colony was transferred with a sterile needle and syringe
5 into a fresh Medium 1 contained in a 30 ml serum bottle. After incubation at 39°C for 24 h the culture was transferred to several slants containing Medium 1 and incubated overnight. These slants were stored above liquid nitrogen for long-term preservation.

4.2 Batch growth rates of isolates in fermenter

10 The growth rates of the isolates were verified using the batch cultivation technique and monitoring the increase in optical density over time. The natural log of the optical density (OD) was plotted against time and the linear part of the graph was used to determine the slope, which represented the maximum growth rate of the organism. Determination of the batch growth
15 rate was performed in a chemostat culture, which was diluted with a sterile medium until a very dilute culture suspension was obtained and the medium supply cut off in order to start the batch growth. The advantage of using a chemostat culture for this work is that there is no lag phase since the cells are all viable and adapted to the medium.

4.3 Analytical techniques

Volatile fatty acids were determined by gas chromatography with a Carlo Erba GC4200 gas chromatograph with flame ionisation detector and a Tupelo 1-1825 column (Supelco Inc., Bellefonte PA, USA). Operating conditions were as follows: carrier gas, nitrogen; flame gases, hydrogen and air; column temperature 175°C; injection port temperature 200°C. A Barspec data system (Barspec Systems Inc., Rehovot, Israel) was used for peak integration. Pivalic acid served as the internal standard. The utilisation of the D- and L-lactate isomers were determined enzymatically (Test combination 10 1112 821, Boehringer Mannheim GmbH, Mannheim).

5. Isolations of bacteria via spread plate method

5.1 Culture media

The incubated rumen fluid lactate (IRFL) medium for the spread plate isolations consisted of 400 ml incubated clarified rumen fluid (Olumeyan *et al.*, 1986) from lucerne-fed sheep, 371 ml distilled water, 2 g peptone (Merck), 15 g agar, 100 ml 10% (w/v) sodium-D, L-lactate solution, 100 ml 0.04% (w/v) bromocresol purple solution and 25 ml mineral solution containing 40 g/l KH₂PO₄; 120 g/l (NH₄)₂SO₄; 8 g/l MgSO₄.7H₂O and 2.4 g/l CaCl₂.2H₂O. Lactic acid (90% w/v) was used to adjust the pH to 5.5 before autoclaving at 121°C for 25 minutes. After sterilisation the medium was cooled down in a 50°C water bath

- while being gassed with an anaerobic gas mixture. Two millilitres of each of the reducing agents, $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ (12.5% w/v) and cysteine. $\text{HCl} \cdot \text{H}_2\text{O}$ (12.5% w/v) were added aseptically. As IRFL medium is not completely selective for lactate-utilisers, bromocresol purple was incorporated to facilitate detection of 5 lactate-utilisers. When lactate is utilised there is a change in ionic balance in the immediate vicinity of the colony causing a pH increase. A rise in pH above 6.3 was indicated as a colour change from yellow to purple in the zone concentric with the culture.
- 10 Acid tolerance was determined on IRFL agar plates with initial medium pH values of 4.5, 5.0 and 5.5.
- Resistance to ionophores was tested on IRFL agar plates containing 10 ppm of ionophores generally used in high-concentrate diets i.e. monensin (Sigma) and 15 lasalocid (Sigma). Repression of lactate utilisation by soluble sugars was tested on IRFL agar plates supplemented with maltose or glucose at a final concentration of 10 g/l. A positive result i.e. a purple zone concentric with the colony indicated that the rate of base release due to lactate utilisation exceeded that of acid production from the added sugar. The isolates were also screened 20 on IRFL agar medium without lactate, but to which glucose or maltose had been added at a concentration of 10 g/l, to determine utilisation of the two sugars.

Growth rates on maltose and glucose were determined on media similar to SDL medium, but in which the lactate was replaced with either 10 g/l glucose or maltose.

5 5.2 Spread plate isolations and screening

The samples for the spread plate isolations were diluted (Mackie & Heath, 1979) in an anaerobic cabinet. Spread plates of IRFL medium were prepared with the 10^{-4} to 10^{-6} dilutions and incubated anaerobically at 39°C. After 24 hours, well-spaced colonies showing a purple zone were transferred to IRFL liquid medium in 1.5 ml microtubes. The inoculated microtubes, which showed a colour change to purple within 16 hours, were screened for acid tolerance, ionophore resistance, catabolite repression and utilisation of glucose and/or maltose. Screening was done by replica plating (Lederberg & Lederberg, 1952) using a multipoint inoculator to inoculate 20 isolates onto a set of nine agar plates of different compositions described above.

5.3 Growth rate determinations

Growth was measured, in triplicate, in SDL, SDG or SDM medium as an increase in turbidity at 578 nm. Vials were incubated in a water bath at 39°C between readings. Readings were continued until the turbidity reached the limit of a satisfactory relationship with biomass. The natural logarithms of optical

density (OD) were plotted against incubation time. The slope of the exponential growth phase that represents the specific growth rate was calculated by linear regression with the aid of a spreadsheet software package.

- 5 The cultures grown on SDL medium at pH 5.7 were then incubated further for a total of 24 hours after which 9 ml was preserved by the addition of one millilitre 10% (w/v) NaOH for analysis of end-products formed and utilisation of lactate isomers.

5.4 Growth physiological studies of plate isolates

- 10 *Fermenter description.* A continuous culture system was set up with three fermenters with a capacity of about 250 ml each. A single peristaltic pump was used to supply medium at different rates to the three fermenters. Temperature of the cultures was maintained at 39°C. The medium and fermenters were 15 gassed with 100% CO₂ to maintain an anaerobic environment. The pH of the cultures was maintained at pH 5.5 by addition of 20% (w/v) orthophosphoric acid as required. The dilution rate was set at 70%, 80% and 90% of maximum growth rate. A sample of 80 ml was withdrawn aseptically from each fermenter at steady state. From this sample the dry mass of the cells and the residual 20 lactic acid in the medium was determined. The biomass output rate, a product of the dilution rate and steady-state biomass, was calculated using the actual dilution rate and the dry mass figures. The growth yield coefficient, which is a function of the biomass concentration at steady state over the amount of

substrate utilised, was calculated using lactic acid residue and dry mass figures.

6. Evaluation of isolate CH4 in high producing dairy cows

5 6.1 Cultivation of lactate utiliser for the animal trial

A Braun Biostat B fermenter with a working volume of 10 litres was transformed into a chemostat using a Watson-Marlow 505S dosing pump equipped with a 55 rpm drive for transporting sterile medium from 50 litre stainless steel kegs.

The working volume was kept constant by continuously transferring excess of culture above the 10-litre level of the fermenter via a dip tube and a peristaltic pump (Watson-Marlow 505S) to a 50 litre polypropylene Carboy that was cooled in a chest freezer. The delivery rate of this harvest pump was set to approximately 120% of the medium supply pump. The excess of volume removed from the fermenter consisted of anaerobic gas from the headspace.

15 A tangential flow filter system (Millipore Pellicon) equipped with a Millipore HVMP 0.45 micrometer (15 ft^2) filter and a Millipore Masterflex Easy-Load peristaltic pump, was used for concentrating the culture.

The medium used was CSL4. The vitamin, reducing agent, mineral and trace element solutions were filter sterilised prior to addition to the medium reservoir.

20 Following autoclaving, the reservoir was gassed with anaerobic gas.

The production approach was a staggered type of production. Two consecutive productions were performed, each producing enough cells for the treatment of one day's group of animals. The number of concentration steps was limited to one per production as each day's production was collected into a 50 litre vessel. The dilution rate of the culture was 0.4 h^{-1} and the "down time" between batches was 50 minutes. A backup run consisting of 45 litres was started prior to the first day's production, which also served to promote the chemostat culture into steady state.

10 6.2 Experimental animals

Sixty high producing dairy cows were blocked according to milk production during previous lactation and body weight and thereafter randomly allocated, within each block, to one of the following treatments: 1) Control diet 60% concentrate; 2) Control diet 60% concentrate + CH4; 3) Control diet 70% concentrate; 4) Control diet 70% concentrate + CH4. Cows were dosed with organism CH4 at calving, 10 days post partum and 20 days post partum.

The following parameters were monitored:

- 20 1. dry matter intake daily;
2. milk production daily;
3. milk fat, protein and lactose weekly; and
4. body weight and condition score monthly.

7. Statistical analyses

Data were analysed by analysis of variance for a completely randomised block design using the program Genstat 5. Previous lactation milk productions were used as a covariate and milk production was reported as covariate adjusted values. Contrasts were used to determine the significance of difference among treatments as follows:

- 10 +CH4 or -CH4 (dosed vs. non-dosed)
 Control diet 70% concentrate vs. control diet 70% concentrate +CH4
 Control diet 60% concentrate vs. control diet 60% concentrate +CH4

Differences were declared significant at P<0.10 and trends were declared at
15 P<0.15 unless otherwise noted.

8. Identification of isolates using phylogenetics, based on 16S rRNA gene sequences.

20 8.1 Bacterial isolates and culture conditions.

M. elsdenii isolates CH4 and CH7, originally isolated from dairy cows (Wiederhold, 1994) were provided by the inventors. The type strain of *M.*

elsdenii, ATCC 25940, was obtained from the American Type Culture Collection. The strains were cultivated in SDL medium as described previously and presumptively identified as *Megasphaera elsdenii* (Wiederhold, 1994).

5 8.2 Amplification and sequencing of 16S ribosomal RNA genes.

Genomic DNA was extracted from bacterial cells using standard procedures (Ausubel *et al.*, 1988). The primers used to amplify the 16S rRNA genes were selected from universally conserved regions in all eubacteria (Table 1). PCR was carried out using primers FD1 (covering positions 8 to 26) and R11 (positions 1384-1400). All target positions of primers used for amplification and sequencing refer to the *E. coli* numbering system (Brosius *et al.*, 1978). The PCR reaction mixture of 100 µl contained approximately 200 ng of DNA, 1 µM of each primer, 200 µM of each nucleotide (dATP, dCTP, dGTP and dTTP), 50mM KCl, 10mM Tris-HCl (pH 8.4) and 2.5mM MgCl₂ and 2.5 U of Taq polymerase (Boehringer Mannheim, Germany). The mixture was overlaid with liquid paraffin to prevent evaporation. The thermal profile consisted of 30 cycles of denaturation for 1 minute at 94°C, annealing at 45°C for 2 minutes and subsequent extension at 72°C for 3 minutes in a thermal cycler (Hybaid, U.K). Final extension was carried out at 72°C for 6 minutes. The homogeneity of the amplicons was analysed by agarose gel electrophoresis (Sambrook *et al.*, 1989). The PCR product was excised from the gel and purified using the Wizard PCR Preps kit (Promega, U.S.A.) as prescribed by the manufacturer.

Direct sequencing of double stranded PCR amplicons and subsequent separation of sequencing reaction products on polyacrylamide gels were essentially carried out according to the protocol of Dorsch and Stackebrandt (1992). Sequencing primers are listed in **Table 1**.

5

Table 1: Primers used to amplify and sequence the 16S rRNA gene. Primer sequences have been published previously (Dorsch and Stackebrandt, 1992; Lane *et al.*, 1985; Stackebrandt and Charfreitag, 1990; Hutson *et al.*, 1993). A 10 combination of these primers covered a total of 1419 nucleotides of the 16S rRNA gene.

Primer Target position ^a Primer sequence (5' to 3')	Primer Target position ^a Primer sequence (5' to 3')	Primer Target position ^a Primer sequence (5' to 3')
<u>Reverse direction</u> (antisense)		
R11 (PCR)	1384-1400	CGGTGTGTACAAGGCC
R1193	1174-1192	CGTCATCCCCGCCTTCCTC
R1353	1336-1352	CGATTACTAGCGATTCC
R961/R7	949-963	TCGAATTAAACCACA
R5	786-802	CTACCAAGGGTATCTAAT
R361/R1	340-355	CTGCTGCCTCCGTAGG

<u>Forward direction</u> (sense)		
FD1/F1 (PCR)	8-26	AGAGTTGATCCTGGCTCA
F1353	1336-1352	GGAATCGCTAGTAATCG
F361	340-355	CCTACGGGAGGCAGCAG
F961	949-963	TGTGGTTAACCGA

^aAll target positions for primers refer to *E. coli* numbering systems (Brosius *et al.*, 1978).

8.3 Data analysis.

- 5 The 16S rDNA sequences obtained were automatically aligned with sequences obtained from the Ribosomal Database Project (RDP; Maidak *et al.*, 1996) using the alignment program CLUSTALW (Genetics Computer Group, 1991). Sequences in the profile were trimmed in order to standardise with regard to the size of the sequences of each organism included in the alignment profile. A 10 total of 1388 nucleotide sequence positions were included in the profile. Published sequences of a number of organisms occurring in the rumen were included in the alignment profile (Table 2). Ambiguous sequences in the alignment profile were manually aligned using the Genetics Data Environment (GDE) alignment editor (Smith, 1992). For inferring phylogenetic relationships, 15 the program fastDNAML (Olsen *et al.*, 1994) was used, which is based on the maximum likelihood algorithm (Felsenstein, 1981). A phylogenetic tree was

constructed using the program TreeTool (GDE). *Escherichia coli* and *Acinetobacter calcoaceticus* served as out groups in the construction of the tree.

5 **Table 2:** Organisms included in the alignment profile using the programs CLUSTALW. All the sequences were retrieved from the RDP and the Genbank databases.

<i>Lactobacillus ruminis</i> ATCC 27780	<i>Streptococcus bovis</i> ATCC 33317
<i>Fibrobacter succinogenes</i> S85 ATCC 1916	<i>Methanobrevibacter ruminantium</i> ATCC 35063
<i>Megasphaera elsdenii</i> ATCC 17752	<i>Methanobacterium formicicum</i> DSM 1312
<i>M. elsdenii</i> ATCC 25940	<i>Methanosarcina barkeri</i> DSM 1538
<i>M. elsdenii</i> CH4	<i>Methanomicrobium mobile</i> ATCC 35094
<i>M. elsdenii</i> CH7	<i>Prevotella ruminicola</i> ATCC 19189
<i>M. cerevisiae</i>	<i>Wolinella succinogenes</i> ATCC 33913
<i>Synergistes jonesii</i>	<i>Escherichia coli</i>
<i>Clostridium acetobutylicum</i> ATCC 824	<i>Acinetobacter calcoaceticus</i> ATCC 33604
<i>Eubacterium cellulosolvens</i> ATCC 43171	<i>Quinella ovalis</i>
<i>Eubacterium uniformis</i> ATCC 35992	<i>Selenomonas ruminantium</i> GA192
<i>Clostridium polysaccharolyticum</i> ATCC 33142	<i>Eubacterium limosum</i> ATCC 8486

RESULTS

Auxostat isolations

5 Isolation of lactate utilising bacteria in auxostat

Isolation 1: The rumen contents, obtained from cow 8710, filling the culture vessel of the fermenter were immediately exposed to fresh sterile selective medium when the auxostat was triggered by an increase in pH. Initial dilution rates were in the region of 0.53 h^{-1} for the first 2 hours at pH 5.30. During the following two hours the dilution rate increased to 0.65 h^{-1} . In order to increase the specificity of the isolation the pH was decreased to pH 5.0, which resulted in a decrease in dilution rate to 0.37 h^{-1} . Cultivation was continued for a further 24 hours after which time only two morphological types could be detected in the enrichment culture. The dilution rate decreased slightly as the cultivation time increased after the initial 24-hour period and at the end of the isolation the dilution rate was only 0.33 h^{-1} .

A sample of the fermenter contents was streaked onto agar medium, in the anaerobic cabinet, and a single colony containing a pure culture was transferred to agar slants and preserved over liquid nitrogen and this culture was denoted as Isolate CH1.

Isolation 2: During this isolation from rumen contents of cow 8812 a dilution rate of 0.25 h^{-1} was observed for the first 24 hours and during the subsequent 24-hour period the dilution rate was between 0.34 and 0.41 h^{-1} . After 48 hours of cultivation it was not clear whether a "pure" culture had been obtained and the cultivation proceeded for a further 24 hours. The dilution rate during this period was 0.41 h^{-1} and a pure culture was isolated from the fermenter via a colony from a Petri dish. This isolate was designated Isolate CH2.

Isolation 3: The dilution rate during this isolation period decreased from 0.28 to 0.21 h^{-1} over a 48-hour period. The isolate obtained from the rumen of cow 8708 was designated Isolate CH3.

Isolation 4: Initial dilution rates were in the order of 0.38 h^{-1} , but within 4 hours the dilution rate decreased to 0.276 h^{-1} and at the end of the 48-hour period the dilution was only 0.197 h^{-1} . The isolate obtained during this isolation from the rumen content of cow 8826 was designated CH4.

Isolation 5: At the end of the isolation period a spore former was the dominant organism and the experiment was terminated.

20

Isolation 6: During this isolation the dilution rate decrease following the same pattern as for the other isolations and the final dilution rate was only

0.116 h⁻¹. The isolate was obtained from rumen contents of feedlot cattle and was designated CH6.

Isolation 7: The rumen contents used during this isolation were obtained from
5 feedlot cattle. The dilution rates decreased from 0.142 to 0.106 h⁻¹, during the first seven hours of isolation. The isolate obtained was designated CH7.

Medium modification for chemostat studies

A consistent decrease in dilution rates was observed during the isolation of the
10 lactate utilisers, which indicated that the formulation of the medium was not optimal. During the first 24 hours of isolation 7 the dilution rate decreased from 0.142 to 0.106 h⁻¹. A pulse dose of 5 ml sterile rumen fluid was added directly into the fermenter and after 4 hours the dilution rate peaked at 0.408 h⁻¹. Thereafter the dilution rate slowly decreased to 0.15 h⁻¹. This "pulse and shift"
15 technique demonstrated that the medium was nutritionally deficient.

Another "pulse and shift" experiment with 1 ml vitamin solution resulted in a dilution rate peak of only 0.28 h⁻¹. However, a larger vitamin pulse resulted in a dilution rate peak of 0.497 h⁻¹, which was higher than with the rumen contents pulse. Lactate utilisation reflected the same results namely, respective D- and
20 L-lactate isomer utilisation of 22 and 86% without extra vitamins, and with extra vitamins respectively 68 and 91%. Medium 1 listed in the Methods reflect the

modified version with higher concentrations of the vitamins. During another "pulse and shift" experiment it was established that yeast extract increased the cell yields of the isolates.

Growth rates of *Megasphaera elsdenii* A TCC 25940 and auxostat isolates

5 vs. pH

Growth rates of the bacteria were determined with the pH-auxostat at various pH values between 4.5 and 6.5, using the modified lactate medium. These growth rates were checked against the values obtained during batch cultivation at the specific pH values and the average value was used.

10 *Megasphaera elsdenii* ATCC 25940, the type strain, showed an increase in growth rate from pH 4.5 up to pH 6.0, followed by a rapid decrease in growth rate at pH 6.5 (Fig. 1). The maximum growth rate achieved by ATCC 25940 was 0.66 h^{-1} , which corresponds to the reported growth rate of 0.6 h^{-1} by Therion *et al.* (1981).

15

All the isolates outperformed ATCC 25940 as far as maximum growth rate was concerned, especially at pH values of 5.5 and below (Fig. 1). The maximum growth rates of the isolates all peaked at pH 5.5 with respective growth rates, (h^{-1}) of 0.66, 0.93, 0.938 and 0.864 for isolates CH7, CH6, CH4 and CH3. Of all 20 the isolates, CH4 proved to be the most acid tolerant with a growth rate of 0.389 h^{-1} at pH 4.5, and the organism with the second best acid tolerance,

CH6, with a growth rate of only 0.19 h^{-1} at pH 4.5. A sharp decrease in growth rate between pH 5.5 and 6.0 was observed for three isolates, namely CH6, CH4 and CH3. Isolate CH7 had only a slight variation in growth rate between pH 5.0 and 6.0, which resembles ATCC 25940 between pH 5.5 and 6.5.

5

Growth rates of auxostat isolates on glucose

The growth rates of three isolates were determined at pH 5.0, 5.5 and 6.0, using the fed-batch growth technique (Fig. 2). Growth rates were noticeably lower for all three isolates on glucose compared to lactate. The most promising 10 isolate on lactate, namely CH4 achieved a maximum growth rate of only 0.25 h^{-1} at pH 5.5 on glucose, compared to 0.938 h^{-1} on lactate. Isolate CH7 achieved the highest growth rate (0.33 h^{-1}) on glucose at pH 6.0 amongst the isolates.

Conversion of lactate by Isolate CH4

15 Isolate CH4 was cultivated at three chemostat dilution rates, namely 0.94, 0.83 and 0.75 h^{-1} on lactate medium. During steady state, samples were taken and analysed for volatile fatty acids (VFA) and the utilisation of lactate determined. Batch cultivation was also conducted and samples were taken at stationary phase. Samples of the sterile medium were also analysed for VFAs and lactate.

20

With an increase in dilution rate the relative production of fatty acids changed, namely at low dilution rates more butyrate and valerate were produced and less propionate and acetate (**Table 3**). At the highest dilution rate very small amounts of butyrate were produced, with no valerate and only 5 slightly more acetate and propionate. Lactate utilisation decreased as expected, with an increase in dilution rate. During the cultivation $D = 0.75$ more than 40% of the lactate was converted to VFAs and although lactate utilisation was high, a large proportion of the available energy was wasted.

When CH₄ was cultivated in batch it produced mainly acetate and 10 propionate. The concentrations of VFAs produced during batch cultivation were much lower than expected and the only explanation would be that CH₄ utilises the VFAs when lactate is depleted.

Table 3: Volatile fatty acids produced by isolate CH₄ from lactate during 15 chemostat cultivation at various dilution rates and during batch cultivation.

Dilution rate (h ⁻¹)	Volatile fatty acids (mM)				% Lactate utilised
	Acetic	Propionic	n-Butyric	n-Valeric	
0.75	7.221	5.779	11.347	6.383	92.66
0.83	10.048	12.293	0.423	0.012	53.54
0.94	8.529	10.517	0.271	0	39.65
Batch	10.659	7.737	0.266	0	97.62

Spread plate isolations and screening

- More than 800 colonies from nine rumen samples of four dairy cattle and two feedlot cattle were inoculated into IRFL liquid medium in microtubes. Of these 610 produced a colour change to purple in the medium within 16 hours of incubation. Nineteen of the screened isolates were chosen for further characterisation as they met the specifications required.
- 10 Four of the selected isolates, AW09; AW10; AW11 and AW12 were capable of growth at an initial pH of 4.5. The other fifteen isolates all grew at an initial pH of 5.0 and there was further selection here, as those cultures showing suitable characteristics, being the fastest growers at pH 5.0, were selected.
- 15 All nineteen isolates were resistant to the ionospheres monensin and lasalocid at concentrations of 10 ppm, utilised lactate in the presence of maltose and glucose and were capable of growth on both glucose and maltose. These nineteen isolates were all Gram-negative cocci (± 1.8 micrometers) occurring in pairs or chains.

Physiological characterisation of isolates

All the AW isolates used both the D- and L-lactate isomers as both isomers were virtually completely utilised after incubation in SDL medium for 24 hours.

Results indicated that the isolates comprised a fairly uniform group and therefore only certain isolates were chosen for further characterisation. For five of the AW isolates the growth rates on glucose at pH 5.8 ranged from 0.38 to 1.05 h^{-1} with a mean of 0.66 h^{-1} (± 0.298). The AW isolates tested for VFA production from DL-lactate were found to produce acetic, propionic, n-butyric and n-valeric acids in the following ratio 2 : 1.5 : 1 : 1.3. Some of the AW isolates produced trace amounts of methyl butyric acid. The maximum biomass output rates obtained for the nine isolates ranged from 0.31 to $0.43 \text{ g (l.h)}^{-1}$. AW15 had the highest biomass output rate and CH4 and AW01 were next in line with $0.39 \text{ g (l.h)}^{-1}$. The yield of cell dry mass per gram of lactic acid utilised, ranged from 0.1 to 0.17 for the nine isolates.

15 Presumptive identification of isolates

The isolates obtained were presumptively identified and those meeting the morphological typing as strains of *Megasphaera elsdenii* were used for further characterisation.

Evaluation of isolate CH4 in high producing dairy cows

The most relevant production data is presented in **Tables 4 and 5**. Data was analysed separately for all cows (15/treatment) and high producers 10/treatment), respectively.

5

Table 4: Effect of organism CH4 on productivity of lactating dairy cattle from calving to 80 days post partum (all cows).

Parameters	Treatment ¹				Contrast P<		
	1	2	3	4	+CH4 vs -CH4	1 vs 2	3 vs 4
Cows per treatment	15	15	15	15	-	-	-
Dry matter intake kg/d	24.6	24.1	23.1	22.2	0.28	0.59	0.32
Milk (kg/d)	36.4	34.0	33.8	32.2	0.10	0.16	0.34
Fat (%)	3.27	3.29	3.57	3.23	0.17	0.85	0.03
Protein (%)	3.10	3.10	3.14	3.07	0.43	0.93	0.23
Body weight	662	608	618	612	0.02	0.004	0.73
Condition score	2.80	2.48	2.45	2.28	0.06	0.08	0.36

10 Treatment 1: Control diet, 70% concentrate + CH4

: Treatment 2: Control diet, 70% concentrate – CH4

Treatment 3: Control diet, 60% concentrate + CH4

Treatment 4: Control diet, 60% concentrate – CH4

Table 5: Effect of organism CH4 on productivity of lactating dairy cattle from calving to 80 days post partum (high producers).

Parameters	Treatment ¹				Contrast P<		
	1	2	3	4	+CH4 vs -CH4	1 vs 2	3 vs 4
Cows per treatment	10	10	10	10	-	-	-
Dry matter intake kg/d	24.6	25.4	24.3	22.6	0.44	0.43	0.06
Milk (kg/d)	39.3	35.9	35.2	34.8	0.13	0.06	0.82
Fat (%)	3.23	3.24	3.56	3.21	0.20	0.91	0.06
Protein (%)	3.10	3.10	3.15	3.02	0.28	0.93	0.11
Body weight	644	597	623	625	0.11	0.02	0.90
Condition score	2.71	2.26	2.34	2.44	0.20	0.02	0.61

5

Identification of isolates using phylogenetics, based on 16S rRNA gene sequences.

Comparative sequencing results showed that our isolates, which are 10 representatives of a larger phenotypically homogenous group, are between 97 and 99% similar (Table 6). Table 7 outlines positions of signature nucleotides suitable to distinguish the two recent *Megasphaera elsdenii* isolates and the ATCC strains from each other. Insertions and deletions accounted for 22% of

nucleotide differences between the four strains. The major nucleotide sequence differences between the strains occur at nucleotide positions 529-536 and 1105-1120 (Table 7). The high sequence similarity displayed between the different *M. elsdenii* strains is also consistent with their similar phenotypic characteristics (Wiederhold, 1994), which is furthermore reflected by their tight phylogenetic clustering. Strains of the species *M. elsdenii* share only 91 to 92% sequence similarity with *M. cerevisiae*, and the two species form distinct clusters in the phylogenetic tree. The *M. elsdenii* cluster bifurcates into two monophyletic groups that evolved from the same ancestral taxonomic unit (ATU). The ATU from which *M. cerevisiae* evolved, however, predates the one from which the *M. elsdenii* cluster evolved. The short branch lengths between *M. elsdenii* strains (OTU's) and their respective ATU's also indicate that they have evolved more recently than the more deeply branched *M. cerevisiae*.

15 **Table 6:** Sequence similarity matrix of 16S rDNA sequences of *M. elsdenii* and *M. cerevisiae*. Sequence similarity values are based on a comparison of a total of 1388 unambiguously aligned nucleotide positions. The %G+C refer only to the respective aligned 16S rDNA sequences.

	ATCC 17752	ATCC25940	CH7	CH4	%G+C
<i>M. cerevisiae</i>	92.0	92.0	91.5	91.5	54.3.
ATCC 25940	99.0		98.5	98.1	54,4
CH4	98.2	98.1	99.0		54.9

CH7	97.7			54.8
ATCC 17752				53.1

Table 7: Sequence signatures defining different *M. elsdenii* isolates and strains

		Nucleotides		
Position ^a	ATCC 25940 ^b	CH4	CH7	ATCC 17752
87	G	G	A	G
105	C	T	C	T
170	T	C	C	T
221	T	C	C	T
241	G	A	A	G
283	A	G	G	A
418	A	*	*	*
529-530	CG	*	*	CG
533-536	CG**	CGAC	CGAC	GC**
539	T	C	C	T
550-552	TAC	CGT	CGT	TAT
556	G	A	A	G
711	G	G	G	C
718	*	*	*	G
850	A	A	G	A

1084	A	G	A	A
1105-1108	TGGA	AGGG	AGGG	TGGA
1117-1120	TCCA	CCCT	CCCT	TCCA
1290	A	*	A	*
1297-1300	AAGT	CGGC	AAGT	CGGC
1396	A	C	A	A
1425	A	A	G	A
1437	G	A	G	G
1492	T	C	C	T

^a *E. coli* numbering system (Brosius *et al.*, 1978). ^bdenotes the type strain. An asterisk indicates where a gap was introduced during the alignment as a result of the occurrence of a nucleotide deletion or insertion at any one position of the sequences of the respective isolates and strains.

The maximum likelihood method, which involves finding a tree, which gives the highest probability of giving rise to the observed sequence data (Felsenstein, 1981), was used to infer a phylogenetic tree from the sequences included in the alignment profile (Fig. 3). This method has the advantage above traditional parsimony methods, which could lead to inference of erroneous trees if different lineages evolve at unequal rates, in that it allows for evolutionary rates to differ between different lineages (Felsenstein, 1981). Since tree topology is also affected by the number of organisms used and the selection of the outgroups

(Stackebrandt and Ludwig, 1994; Stackebrandt and Rainey, 1995), a number of apparently related and apparently unrelated organisms, occurring in the rumen was included in an alignment profile. This was subsequently used to construct the tree. Although the phylogenetic tree was inferred from only nearly 5 complete (92%) 16S rRNA gene sequences, which could reduce resolution between very closely related organisms (Utaker *et al.*, 1995; Li and Graur, 1991), the general topology of trees derived from either complete or partial sequences has been shown to be in overall agreement with each other (Van Camp *et al.*, 1993, Vandamme *et al.*, 1996).

10

Vandamme *et al.* (1996) proposed that different isolates should be regarded members of the same species if they share more than 97% rRNA sequence homology, show phenotypic consistency and exhibit a significant degree of DNA: DNA hybridisation. Although the relationship between DNA similarity and 15 16S rRNA sequence homology between organisms is anything but linear, Fox *et al.* (1992) proposed that effective 16S rRNA sequence identity should imply that two organisms are members of the same species, since it would almost certainly be validated by the DNA: DNA hybridisation. Although 16S rRNA sequence data alone may not be sufficient in all cases to define a species, it is 20 extremely useful in determining to which species a strain probably belongs, once the relevant species is represented in a 16S rRNA sequence data base. Strains with almost identical 16S rRNA sequences should be assigned to the same "rRNA superspecies" or "rRNA species complex". It would thus be

appropriate to assign isolates CH4 and CH7, phenotypically presumptively identified *M. elsdenii* strains, to the same rRNA species complex, which would include reference strains ATCC 25940, the type strain of the species, and ATCC 17752. Since the phylogenetic relationships of the respective isolates
5 are furthermore consistent with their phenotypic characteristics, these isolates can be considered strains of the species *Megasphaera elsdenii*. The fact that *M. cerevisiae* and *M. elsdenii* share only 92% 16S rDNA sequence homology, confirms, together with genotypic and phenotypic data, the division of the genus into two well resolved species.

10

Of the rumen bacteria included in this study the ones which appear to be most closely related to the *Megasphaera* cluster are *Selenomonas ruminantium* and *Quinella ovalis*. The apparent phylogenetic relationship between *Megasphaera elsdenii* and *Selenomonas ruminantium* is consistent with some phenotypic and
15 genotypic characteristics which the two species share, such as similar DNA G+C content (53-54%), anaerobic nature, chemoorganotrophic metabolism and utilisation of a similar range of substrates (Stackebrandt *et al.*, 1985; Stewart and Bryant, 1988; Haikara, 1992). The work of Stackebrandt *et al.* (1985), who made use of the oligonucleotide cataloguing technique for phylogenetic
20 inference between the species, supports this phylogenetic relationship.
Selenomonas ruminantium on the other hand is also closely related to the relatively unknown *Quinella ovalis*, an organism that proliferates in the rumen when sugar rich diets are fed to the animal. These organisms, although not

- established in culture yet, share some physiological characteristics with the large selenomonads found in sheep (Stewart and Bryant, 1988). As expected, the most distant relatives of *Megasphaera* that occur in the rumen are those contained in the archaeal methanogen cluster, the members of which are believed to have appeared approximately 600 to 800 million years ago (van Soest, 1994; Woese, 1987). The evolutionary rates of these organisms are also slower than that of the *Bacteria*, and the primitiveness of the group is clearly reflected by the deeply branched methanogen cluster.
- The recent divergence of the different *M. elsdonii* strains could possibly be attributed to a refinement of its phenotype in order to adapt to the highly selective conditions in the rumen. According to Woese (1987), the evolution of the phenotype of an organism is a process during which new or more efficient traits are gained in order to survive in its particular niche. Refinement would result in the organisms being metabolically versatile, as is the case with *Megasphaera*. The slower evolving methanogens on the other hand are metabolically monotonous by comparison.

This study has demonstrated the suitability of 16S rDNA sequencing to distinguish between closely related strains of the species *M. elsdonii*. Furthermore, it provided a phylogenetic framework for identification of recently isolated strains that have been characterised phenotypically. The framework

would be of particular value in serving as a basis for the design of species and strain-specific probes intended for rumen ecological studies.

CONCLUSIONS

5

Isolation

The incorporation of bromocresol purple to IRFL medium to facilitate detection of lactate-utilising bacteria proved to be successful in the case of
10 the faster-growing lactate-utilisers, which were of prime interest in this study.

In the early stages of incubation these produced purple zones, concentric with the colonies, which contrasted clearly with the yellowish background of the agar medium. However, on extended incubation the pH gradient surrounding the colonies dissipated due to diffusion of ions and the whole
15 background became purple. Differentiation between colonies of slower-growing lactate-utilisers and those of organisms growing on other carbon sources present in the rumen fluid supplement then became difficult.

M. elsdenii is not the dominant lactate-utilising species in animals on high-concentrate diets (Mackie *et al.*, 1978; Mackie & Gilchrist, 1979; Mackie *et al.*,
20 1984; van Gylswyk, 1990), but there are a number of reasons why they may have predominated in the selection and screening procedures.

Some of the colonies screened consisted of Selenomonads and other morphological types. Most of these colonies were not chosen as there was not a positive indication that lactate could be utilised in the presence of soluble sugars. Russell & Baldwin (1978) showed that *M. elsdenii* B159 used glucose, 5 maltose and lactate, but not sucrose simultaneously in a multi-substrate medium. Marounek *et al.* (1989) showed that for four strains of *M. elsdenii* lactate was used more rapidly than glucose in media with both carbon sources.

Certain other laboratories, which have studied the possibility of inoculating 10 ruminants on high-concentrate diets with lactate-utilising organisms to prevent an accumulation of lactate, have also worked with *M. elsdenii* strains (Das, 1979; Leedle *et al.*, 1991; Robinson *et al.*, 1992; Kung & Hession, 1995; Wiryawan & Brooker, 1995). It was not possible to compare the growth rates of 15 the AW and CH isolates to strains in the literature to determine if they had faster growth rates and if they were more acid tolerant, as no results were available in literature. The AW and CH isolates can however be compared to the type strain, *M. elsdenii* ATCC 25940.

For the AW isolates the range of pH values at which growth was determined 20 was not sufficient to determine the pH range for the isolates or the optimum pH for growth. However, it can be assumed that the optimum would be above pH 5.7 and the lowest pH would lie between pH 4.5 and pH 4.9 for all but four of the isolates; as there was no growth on IRFL plates at pH 4.5. This agrees with

work done on the type strain of *M. elsdenii* ATCC 25940. The pH range for the type strain of *M. elsdenii* ATCC 25940 is pH 4.6 to 7.8 with the optimum for growth at pH 6.05 (Therion *et al.*, 1982).

- 5 For the CH isolates the optimum pH for growth is between pH 5 and 6. In the range of pH values tested, the highest growth rates were found at pH 5.5. Both sets of isolates had higher growth rates on SDL medium than the type strain. The growth rates obtained for *M. elsdenii* ATCC 25940 in SDL medium is comparable to that obtained by earlier workers on lactate medium (Therion *et*
- 10 *al.*, 1982).

The growth rates of the isolates on lactate were higher than on glucose and maltose. This is in agreement with a previous study on *M. elsdenii* ATCC 25940, where growth rates on lactate medium between pH 5.0 and pH 6.5 was 15 found to be higher than in glucose medium (Therion *et al.*, 1982). Outside this pH range growth rates on glucose were higher. A study on substrate preference in rumen bacteria reported that growth of *M. elsdenii* B159 on lactate was slower than on glucose and maltose, however the pH of the media in the study was above pH 6.5, being between 6.75 and 6.9 (Russell & Baldwin, 20 1978).

The composition of fermentation end products on lactate medium has been determined for four strains of *M. elsdenii*, including the type strain LC1 or ATCC

25940 (Marounek *et al.*, 1989). These results showed strain-to-strain variability in the proportions of fatty acids formed. Three of the strains produced little or no valeric acid while 22 mol% of the end-products of *M. elsdenii* L8 was valeric acid (Marounek *et al.*, 1989). The nine AW isolates tested in the present study 5 did not exhibit as much strain-to-strain variability as was the case in the strains tested by Marounek *et al.* (1989), but are similar to *M. elsdenii* L8, which was isolated from the rumen of a calf on a milk diet, as valeric acid was produced. CH4, however, produced the same fermentation end-products as the type strain.

10

From the point of view of maximum biomass output rate in SDL medium strain AW15 would be the organism of choice for larger scale production of cells for animal experiments with CH4 and AW01 being next in line. The time required to produce 100g dry mass of cells on SDL medium in a chemostat of 5l working 15 volume would be 1.9 days for AW15 and 2.1 days for CH4 and AW01.

The growth rates of the selected isolates on lactate are high compared to the type strain of *M. elsdenii*. The isolates are acid tolerant and can grow at pH values below 5.0. They are resistant to ionophores, commonly added to feedlot 20 diets, and can utilise both isomers of lactic acid even in the presence of glucose and maltose. The fermentation end products from lactate are VFA, which are an important energy source for the ruminant. Propionate production is especially important in the feedlot industry, as propionate is the main source of

glucose for the ruminant tissues. The isolates, therefore, have the characteristics required for an effective product to combat lactic acidosis in ruminants.

- 5 Cultivation of the lactate utilisers was successful using a medium that did not contain any rumen fluid. The only modification to the original medium was the increase in the vitamin content and the addition of yeast extract to the medium. Bacteria survived remarkably well on this medium at 4°C for up to 20 days, when used as working cultures.

10

The technique of using a pH-auxostat for the enrichment of lactate-utilising rumen bacteria, with a predetermined combination of biochemical/physiological attributes, which would make them potentially highly suitable for preventing and combating lactic acidosis in feedlot animals was very successful. In most cases

- 15 a fast-growing, morphologically homogeneous population became established in the fermenter within two days after the start of a run. Subsequent tests on the cultures that were isolated from the fermenter contents by plating, confirmed that the cultures possessed the desired combination of characteristics.

- 20 Presumptive identification of the isolates from the enrichments showed that all but one belonged to the species *Megasphaera elsdenii*.

Table 8: A comparison between the pH-auxostat isolation technique and a conventional spread plate screening technique.

Parameter	Conventional spread plate	Auxostat
Time elapsed (days)	90	9
Man hours spent	180	7
Sample: bacterial load (cfu)	12×10^{10}	14×10^{12}
Maximum specific growth rate (h^{-1})	0.91	0.90
Biomass yield (g.l^{-1})	0.60	0.59
Biomass output rate g(l.h)^{-1}	0.39	0.39

- 5 Since the isolates performed better than ATCC 25940 at pH values below 6.0, they would be more suited to the ruminal pH encountered by feedlot animals that usually is below pH 6.0. Furthermore, CH4 proved to be the best suited isolate for trial experimentation on feedlot animals.

- 10 Cultivation of the lactate utilising isolates on glucose proved not to be a proposition due to the slow growth rates obtained compared to the growth rates obtained on lactate.

The thirty cows that were dosed with organism CH4 (Table 4) produced significantly more milk ($P=0.10$), had a higher average body weight ($P=0.02$) and body condition score ($P=0.06$). The milk fat percentage of cows receiving the 60% concentrate diet + CH4 were also significantly increased (3.57% vs.

5 3.23%).

Animal trials

The data on the 40 high producing cows is presented in Table 5. Dry matter intakes did not differ but milk production was significantly increased by 3.4 kg/d from 35.9 to 39.3 kg/d ($P=0.06$) when cows were fed the 70% concentrate diet and were dosed with organism CH4. Milk production tended to be increased ($P=0.13$) when all dosed cows were compared to all non-dosed cows. Body weight and condition score were increased ($P=0.02$) when high producing cows receiving the high concentrate diet were dosed with organism CH4. The dosing of cows receiving the 60% concentrate diet resulted in a significant increase in milk fat percentage ($P=0.06$) with a tendency towards increased milk protein ($P=0.11$). Milk components play an important role in current milk payment schemes.

20

Dosing cows with organism CH4 significantly increased milk production and positively affected milk composition, body weight and body condition score. Dry matter intakes were not affected; therefore results suggest that dosing of

cows with organism CH4 caused a more favourable rumen environment, which resulted in improved utilisation of nutrients.

The applicants have further found that, in comparison with the known strains of *M. elsdenii*, the *M. elsdenii* CH4 strain is:

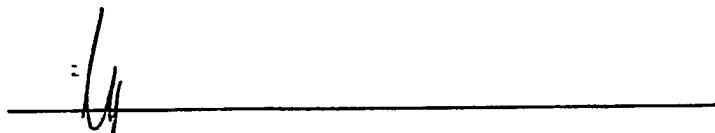
- 5 - highly active and adapted to proliferate in the rumen of animals on high-concentrate diets;
- capable of proliferating at relatively low pH values below pH 5.0 and as low as 4.5, characterised as acute acidosis;
- 10 - resistant to ionophore antibiotics commonly added to feedlot diets; and
- capable of preferentially using lactate as a carbon source even in the presence of soluble carbohydrates such as glucose and maltose.

- 15
- Further advantages of this strain are that it:

- has a relatively high growth rate, i.e. more than 0.938 h^{-1} ;
- 20 - has the ability to grow on reducing sugars as well as on lactate;
- has a relatively high biomass output rate, i.e. more than $0.39 \text{ g (l.h)}^{-1}$;

- is ionophore resistant;
 - produces predominantly acetate and not predominantly propionate and butyrate; and
 - has a unique 16S rRNA sequence and is therefore a new strain.
- 5 The applicants have yet further found that animals challenged with maltose, fed directly into the rumen, produced no measurable build-up of lactate in the rumen when inoculated with CH4.
- Furthermore, high producing dairy cows inoculated with CH4 has a 2.4 to 3.2 litres higher production of milk, than control animals not inoculated with CH4.
- 10 The body condition score as well as body weight of the inoculated cows were statistically significantly higher than the control animals.
- It will be appreciated that variations in detail are possible with a microorganism according to the invention and its uses without departing from the scope of this
- 15 disclosure.

DATED THIS 18.. DAY OF JULY 2002.



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D M KISCH INC

PATENT ATTORNEYS FOR THE APPLICANTS

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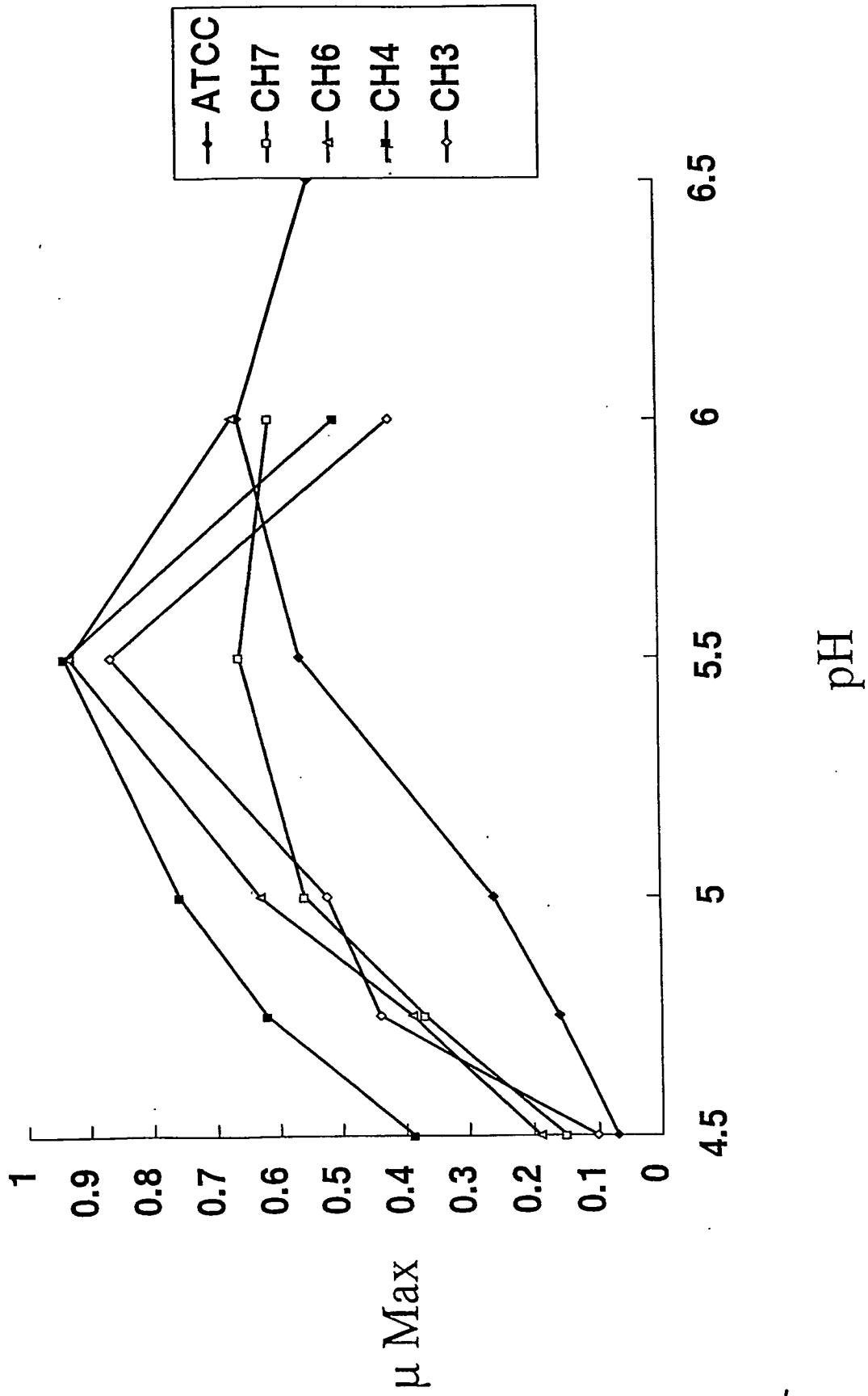


Fig. 1. Growth rates of lactate utilisers at various pH values.

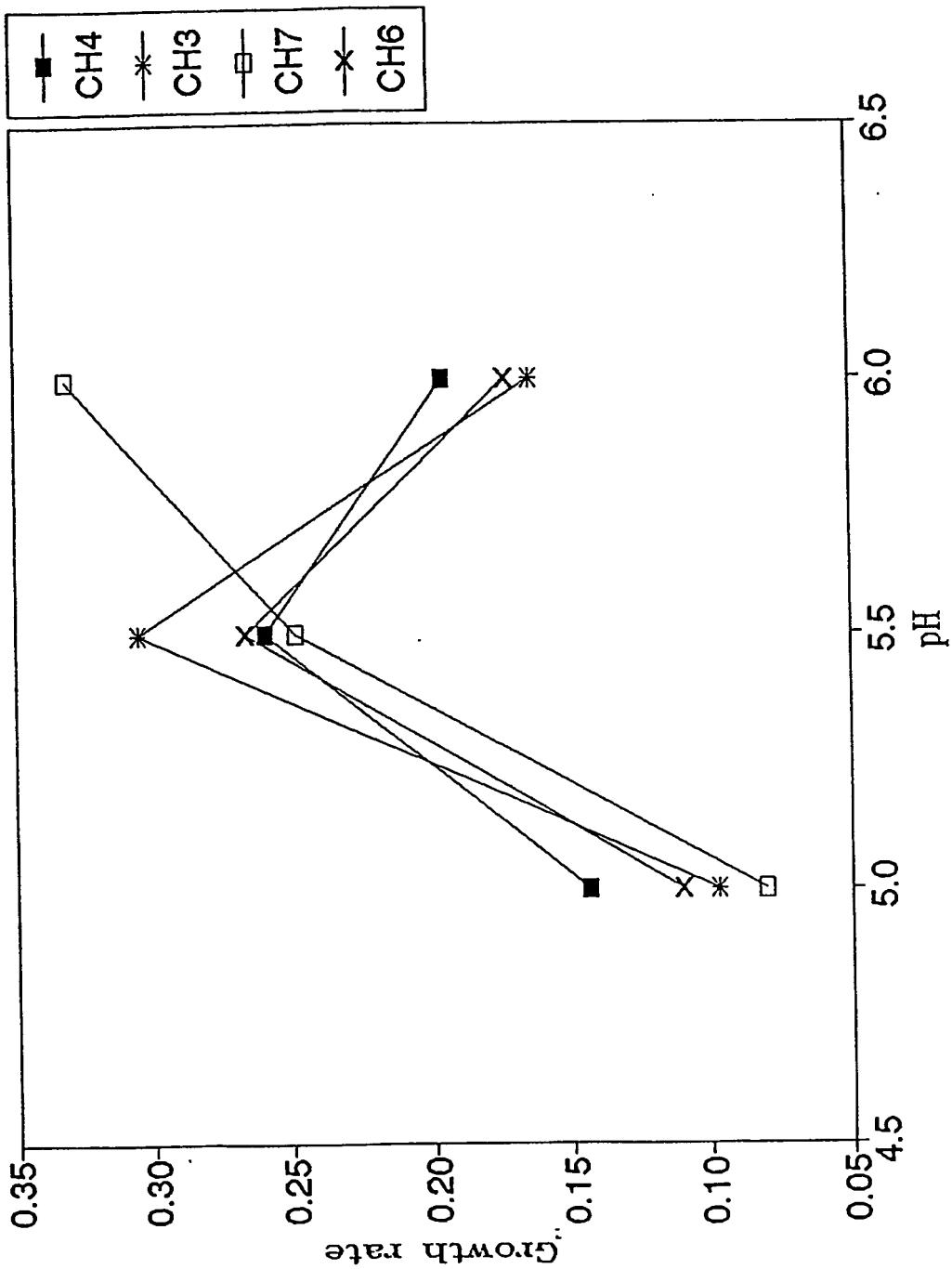


Fig. 2. Growth rates (h^{-1}) of the lactate utilizing isolates, on glucose medium at various pH values.

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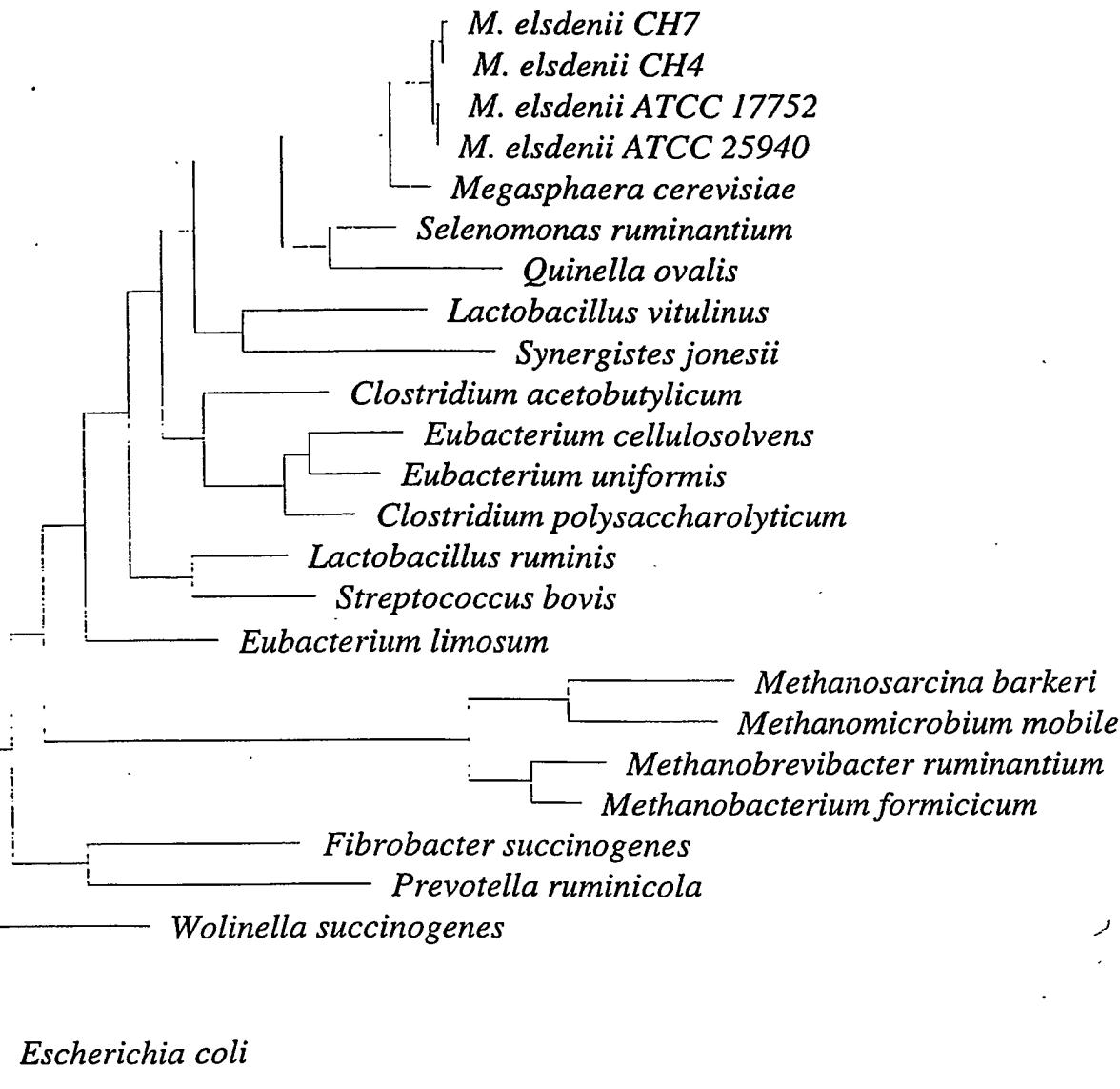


Fig. 3

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